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TITLE: Relaxation of Insulin-Like Growth Factor II Imprinting in
Prostate Cancer Development

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13. ABSTRACT (Maximum 200 Words) A marked propensity for prostate cancer arises in the peripheral prostate with aging. The insulin-like Growth Factor-II (IGF-II) gene is an auto-paracrine growth stimulator that is an important positive modulator of cancer development. IGF-II typically demonstrates monoallelic, or imprinted, expression in adult tissues and indeed this pattern is maintained in the periurethral zone, a region where cancer development is rare. In addition, IGF-II loss of imprinting (LOI), as well as increased IGF-II expression, are common attributes of prostate cancer. It is our hypothesis to be tested that an age-dependent loss of IGF-II imprinting, resulting from age-dependent changes in DNA methylation, occurs specifically in the peripheral zone of the prostate and contributes to the increased risk for cancer development. To examine temporally when this loss of IGF-II imprinting occurs and the mechanisms underlying it we propose 3 Specific Aims: (1) To determine if IGF-II LOI in the peripheral prostate derives from stromal and/or epithelial cells; (2) To determine whether IGF-II LOI occurs as an age-dependent process in human prostate tissue that are uninvolved with cancer; and (3) To examine DNA methylation as a mechanism for any observed changes in the imprint status in prostate tissues. This proposal is significant and unique in testing whether regional epigenetic changes occur in histologically normal prostate tissues that are destined to become neoplastic. We expect to determine whether specific age-related, peripheral zone changes in methylation and imprinting occur in the general population and whether these changes are linked to prostate cancer development.				
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INTRODUCTION

Three important features of prostate cancer will be addressed in the present proposal that may provide a quantum leap in our understanding of the risk factors and development of prostate cancer. These features include: (1) A marked propensity for prostate cancer to arise in the peripheral prostate; (2) The multifocality of prostate cancer which implicates a generalized or field change in cancer susceptibility; and (3)

The important role of the Insulin-like Growth Factor (IGF) axis in both aging-related and genetic-related cancers. The Insulin-like Growth Factor-II (IGF-II) gene is a auto-paracrine growth stimulator that is an important positive modulator of cancer development. We will provide preliminary evidence that a loss of imprinting, or biallelic expression, of the IGF-II gene is an age-related specific epigenetic alteration that occurs in the peripheral prostate. IGF-II typically demonstrates monoallelic, or imprinted, expression in adult tissues and indeed this pattern is maintained in the periurethral zone, a region where cancer development is rare. In addition, IGF-II loss of imprinting (LOI), as well as increased IGF-II expression, are common attributes of prostate cancer. Since DNA methylation is the major determinant of gene imprinting, we would anticipate that a loss of IGF-II imprinting in the prostate will be associated with specific changes in the IGF-II/H19 promoter regions.

It is our hypothesis to be tested that an age-dependent loss of IGF-II imprinting, resulting from age-dependent changes in DNA methylation, occurs specifically in the peripheral zone of the prostate and contributes to the increased risk for cancer development.

To examine temporally when this loss of IGF-II imprinting occurs and the mechanisms underlying it we propose 3 Specific Aims: (1) To determine if IGF-II LOI in the peripheral prostate derives from stromal and/or epithelial cells; (2) To determine whether IGF-II LOI occurs as an age-dependent process in human prostate tissues that are uninvolved with cancer; and (3) To examine DNA methylation as a mechanism for any observed changes in the imprint status in prostate tissues. We propose to confirm our preliminary observations through a comprehensive analysis of IGF-II LOI and DNA methylation analysis in aging prostate tissues associated with and without cancer. These studies will utilize a unique tissue bank containing normal prostate tissues of various ages. Several innovative techniques including laser capture microdissection and quantitative allele-specific imprinting assays will also be employed.

BODY

Task 1: To determine whether a LOI in IGF-II arises from prostate epithelial cells, stromal cells or both in normal human prostate tissues.

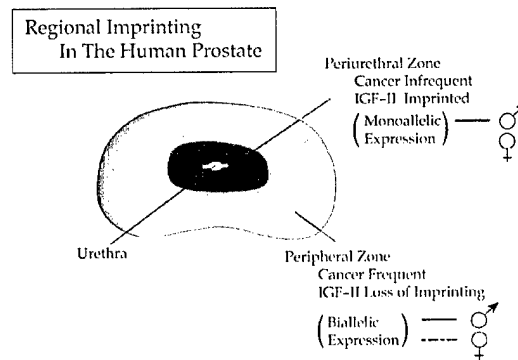


Figure 1: The IGF-II gene demonstrates monoallelic (paternal only) expression in the periurethral zone where benign prostatic hyperplasia originates. This pattern is found in most adult tissues. In the peripheral zone, where prostate cancer arises, expression is from both inherited alleles(4).

1. *Acquisition and histologic analysis of prostate specimens, DNA production (Months 1-3)*: We have histologically examined to exclude concomitant cancer, made DNA and screened over 70 samples for the *Apal* polymorphism. Twenty-five have been found to contain the *Apal* polymorphism. DNA has been extracted from these tissues.
2. *Laser capture microdissection of stromal and epithelial cells from normal peripheral zone prostate tissues and RNA isolation (Months 1-12)*: We have utilized laser capture to separate the stroma from the epithelium in 5 tissues from the peripheral prostate of samples from men in their 60's.
3. *Imprinting analyses using RT-PCR/restriction enzyme digestion and development of quantitative allele-specific PCR assay (Months 2-14)*: We have found that in all 3 samples of normal peripheral prostate analyzed to date, both the epithelium and stroma demonstrate biallelic expression. This is remarkable since the majority of tissues maintain a tight regulation of this gene. The epithelial-specific change suggests a regional epigenetic cell-specific alteration may predispose these cells to the formation of cancer. This would support our hypothesis. Furthermore, this validates the epithelium as the cell of origin for prostate cancer. The stromal expression may function through an inductive (paracrine) effect on the epithelium. Stromal-specific alterations are important in the induction of epithelial cancers and could possibly represent an important clonal change.

Task 2 : To examine the frequency of LOI in IGF-II in aging human prostate tissues containing no associated prostate cancer.

1. *Acquisition of non-tumor associated peripheral zone prostate tissues from different ages and DNA production (Months 10-12):* To increase the number of samples we have taken additional approaches. We have screened a population of samples for additional polymorphisms in exon 9 of the IGF2 gene. We have found two additional polymorphisms including a C/T (266, frequency 10%) and C/G (1926, frequency 30%). These sites do not contain cutting sites for restriction enzymes, thus we have had to develop a new assay for assessing imprinting. Using single-nucleotide primer extension (SNUPE) we are able to accurately detect single nucleotide base changes to detect imprinting changes in tissues. To validate this approach we have examined a polymorphism in a B6(cast IGFII) mouse provided by Dr. Shirley Tilghman. We find the assay to be reproducible and sensitive (Figure 2). To increase the pool of samples we have contacted and obtained permission to analyze a bank of aging, non-cancer tissues at the University of Pittsburgh (Dr. Rob Getzenberg). IRB consent has been submitted to analyze these tissues and they will be incorporated in the next 6 month cycle of the grant.
2. *Microdissection and isolation of RNA (Months 12-24):* We have validated our technique for microdissection and obtaining RNA. DNA

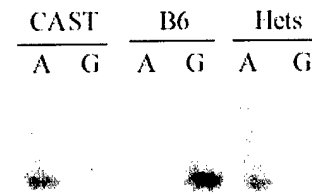


Figure 2: Allele-specific expression is analyzed using the SNuPE assay. Expression of IGF2 is seen to be expressed primarily from the A allele in heterozygous mouse prostate tissue. Some expression (10%) is noted from the G allele suggesting incomplete imprinting in this tissue. This easily quantitated assay is being applied to human tissues.

contamination remains a problem, however we have experimented with multiple approaches and found that a double DNase digestion technique and purification on a Quiagen column negates any detectable DNA that remains.

3. *Imprinting analyses using RT-PCR/restriction enzyme digestion and development of quantitative allele-specific PCR assay and statistical analysis (Months 12-26).*
Because of the need for additional polymorphisms to increase our sample size, we will be utilizing the SNUPE technique for allele analysis as described and shown above.

Task 3: To examine whether methylation alterations underlie differences in IGF-II imprinting in the human prostate.

1. *Microdissection and isolation of DNA from imprinted and loss of imprinting prostate tissues (Months 24-32).*
2. *Treatment of DNA with sodium bisulfite, PCR of CpG-enriched regions and sequencing (Months 24-34)*

We have identified areas within the IGF-II/H19 locus that alter their methylation status with human prostate epithelial cell (HPEC) aging in an *in vitro* model. Senescence is an *in vitro* model of aging that recapitulates many of the gene changes seen in *in vivo* aging. Utilizing collagen-coated plates and a low serum media to exclude fibroblasts human prostate epithelial cells are cultured through 15-20 population doublings before growth ceases and a characteristic senescent morphology and senescence-associated β -galactosidase staining develops. In all epithelial cultures, a complete relaxation of the *IGF-II* imprint was reproducibly found with passage to senescence. One of the strongest candidates for control of the *IGF-II* imprint is an alteration in DNA methylation.

We find that DNA methylation inhibition using 2-deoxy 5'azacytidine leads to an accelerated loss of IGFII imprinting in HPEC cells. In addition, DNA methylation changes occur consistently in aging both *in vivo* and *in vitro*. These changes include both a global hypomethylation as well as regional hypermethylation of selected CpG islands. We have found a consistent increase in DNA methylation in a differentially methylated region in the human, intergenic between H19 and IGF-II, that appears to harbor a methylation imprinting mark for the IGF-II gene. Deletion or hypermethylation of this CpG island has been demonstrated in mouse models to lead to biallelic IGF-II expression. Methylation at this locus will be assessed in *in vivo* human prostate tissues.

We have also found that other epigenetic alterations may play an important role in the loss of imprinting in senescence. CTCF is an insulator protein that binds in the IGF-II/H19 intergenic region. Binding occurs on the nonexpressed allele and is hypothesized to prevent enhancer binding to the IGF-II promoter region. We have found that CTCF expression decreases over 10 fold in senescing prostate epithelial cells and this may result in the biallelic expression seen (manuscript in revision). Expression of this gene will be important to assess in aging prostate epithelial cells as well.

3. *Quantitative methylation-sensitive single-primer extension analysis of specific sites (Months 24-36)*

KEY RESEARCH ACCOMPLISHMENTS

- We have demonstrated that both the epithelium and the stroma express the IGF-II gene from both alleles.
- Inhibition of DNA methylation results in an acceleration of loss of IGF-II imprinting.
- We have analyzed a series of CpG islands in aging epithelial cells *in vitro* and have identified several loci that alter methylation status with increased population doublings.
- A decrease in the expression of CTCF is found at senescence in human prostate epithelial cells which provides a mechanism for the loss of IGF-II imprinting in aging cells.

REPORTABLE OUTCOMES

Manuscripts:

Fu VX, Schwarze SR, Grabert L, LeBlanc S, Svaren J, and **Jarrard DF**. A Loss of IGF2 Imprinting is Modulated by CTCF Downregulation at Senescence in Human Epithelial Cells. (JBC, in revision)

Atwood CS, Barzilai N, Brown-Borg HM, **Jarrard DF**, Fu VX, Heilbronn LK, Ingram DK, Ravussin E, Schwartz RS, and Weindruch R. Pennington Scientific Symposium on Mechanisms and Retardation of Aging, *Exp Gerontology* 38(10)1217-1226, 2003.

Funding: An RO1 (CA97131) was successfully funded based in part on data obtained from this grant. This study will look at age-related changes in imprinting in the mouse prostate and cancer susceptibility. In addition, a program project grant entitled "Modulation of Genomic Imprinting on Oxidative Stress" that seeks to examine whether oxidative stress can modulate the imprinting of IGF-II has been funded through the NIH.

CONCLUSIONS

IGF-II loss of imprinting is found in aging, senescent human prostate epithelial cells consistent with our hypothesis that it is modulated with aging. Both stroma and epithelia from peripheral prostate tissue in older men express biallelic IGF-II. This is in contrast to the tight imprinting in other tissues. DNA methylation plays a role in IGF-II imprinting control, however CTCF expression modulates it in human prostate cells. These findings may be important in the susceptibility of the prostate for developing age-related cancers.